

CLINICAL SCIENCE

The *ADA**2 allele of the adenosine deaminase gene (20q13.11) and recurrent spontaneous abortions: an age-dependent association

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OBJECTIVE: Adenosine deaminase acts on adenosine and deoxyadenosine metabolism and modulates the immune response. The adenosine deaminase G22A polymorphism (20q.11.33) influences the level of adenosine deaminase enzyme expression, which seems to play a key role in maintaining pregnancy. The adenosine deaminase 2 phenotype has been associated with a protective effect against recurrent spontaneous abortions in European Caucasian women. The aim of this study was to investigate whether the G22A polymorphism of the adenosine deaminase gene is associated with recurrent spontaneous abortions in Brazilian women.

METHODS: A total of 311 women were recruited to form two groups: G1, with a history of recurrent spontaneous abortions (N = 129), and G2, without a history of abortions (N = 182). Genomic DNA was extracted from peripheral blood with a commercial kit and PCR-RFLP analysis was used to identify the G22A genetic polymorphism. Fisher's exact test and odds ratio values were used to compare the proportions of adenosine deaminase genotypes and alleles between women with and without a history of recurrent spontaneous abortion ($p < 0.05$). The differences between mean values for categorical data were calculated using unpaired t tests. The Hardy-Weinberg equilibrium was assessed with a chi-square test.

RESULTS: Statistically significant differences were identified for the frequencies of adenosine deaminase genotypes and alleles between the G1 and G2 groups when adjusted for maternal age.

CONCLUSIONS: The results suggest that the adenosine deaminase *2 allele is associated with a low risk for recurrent spontaneous abortions, but this association is dependent on older age.

KEYWORDS: Adenosine deaminase; Recurrent spontaneous abortions; *ADA* gene.

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INTRODUCTION

Recurrent spontaneous abortion (RSA) is defined by two or more consecutive miscarriages before 20 weeks of gestation.^{1,2} The etiology of RSA is varied, but immunological, anatomical, endocrine, and genetic aspects are considered the most important factors. These factors may act on fetus selection in such a way as to impede the implantation of the zygote or to prevent fertilization.³

The enzyme adenosine deaminase (ADA; EC 3.5.4.4) catalyzes the deamination of adenosine and deoxyadenosine, thereby affecting the methylation process, cell growth and differentiation, apoptosis, DNA replication and immune functions.^{4,5} The genetic deficiency of ADA in humans results in severe combined immunodeficiency syndrome (SCID) of both the humoral and cellular immune responses.⁶ The polymorphism of the *ADA* gene (20q13.11) resulting from the substitution of G by A at nucleotide 22 of exon 1 replaces the Asp amino acid (*ADA**1 allele) with Asn (*ADA**2 allele) amino acid in position 8 of the enzyme. Consequently, individuals with the *ADA**2 allele express low levels of ADA compared to homozygous *ADA**1/*1 individuals.⁷

ADA activity is critical in maintaining a normal pregnancy.^{8,9} In a previous report, Nicotra et al.¹⁰ observed that the frequency of the *ADA**2 allele is lower in European

women who suffer RSA than those who do not, suggesting a protective effect of this allele against RSA and higher fertility rates among women with *ADA*2* allele. Recently, these same authors observed a synergic effect of *ACP1* (low molecular weight protein tyrosine phosphatase) and *ADA* polymorphisms with respect to RSA.¹¹ These authors concluded that women with high *ADA* activity and low *ACP1* activity have a higher susceptibility to RSA. A European ethnic background is present in the Brazilian population,¹² but data are scarce on the importance of the *ADA G22A* genetic polymorphism in RSA in Brazilian women. The aim of this study was to investigate whether the *ADA G22A* polymorphism is associated with RSA in Brazilian women.

PATIENTS AND METHODS

Ethical considerations

This study was approved by the Research Ethics Committee of the Medical School in São José do Rio Preto - FAMERP (# 308/2008). The objectives of the investigation and all procedures performed in the study were explained to selected patients, and those who agreed to participate in the study gave their written consent.

Patient selection

Two groups of pregnant women were selected from the Gynecology and Obstetrics Clinic, Hospital de Base from the Regional Medical School Foundation in São José do Rio Preto, São Paulo State, Brazil. The first group (G1, N=129) included only women who had suffered at least two consecutive spontaneous abortions with the same partner according to their medical records and to reports from the patients themselves. The second group (G2, N=182) included only women with at least two successive spontaneously conceived pregnancies and no history of spontaneous abortion. Patients who were younger than 18 years old were excluded from the study.

Data collection

An epidemiological questionnaire was completed by all participating patients, and these data were later confirmed with their medical records. Comorbidities such as a history of diabetes, hypertension, polycystic ovarian syndrome, uterine malformation, antiphospholipid antibodies, and endometriosis were also collected from the medical records.

Collection of blood samples and genomic DNA extraction

Five milliliters of peripheral blood was collected from each participant in tubes containing EDTA anticoagulant. Genomic DNA was extracted from 200 μ L of whole blood using a commercial kit (PureLink™ Genomic DNA Mini Kit, Invitrogen). The manufacturer's instructions were strictly followed.

Identification of the *ADA*1* and *ADA*2* alleles

The identification of the *ADA*1* and *ADA*2* alleles was achieved using PCR-RFLP analysis following the protocol of Safranow et al.¹³ A gene amplification reaction (25 μ L final volume) was performed for each sample of genomic DNA under the following conditions: 7.2 μ L of MilliQ water, 5.0 μ L of PCR Buffer Green (5x, Promega), 1.5 μ L $MgCl_2$ (25 mM,

Promega), 2.1 μ L of DMSO (Nuclear), 1.0 μ L of 2-mercaptoethanol (200 mM, Vetec), 1.0 μ L of sense primer (5 pM, IDT; 5'-GCCCCGCCCCGTTAAGAAGAGC-3'), 1.0 μ L of antisense primer (5 pM, IDT; 5'-GGTCAAGTCAGGGGCAG-AAGCAGA-3'), 4.0 μ L of dNTPs (1.25 mM, Invitrogen), 0.2 μ L of GoTaq Hot Start DNA polymerase (5 U, Promega), and 2.0 μ L of genomic DNA. As an internal contamination control, a tube was prepared under identical conditions but without the genomic DNA (blank). The amplification conditions were as follows: 94°C for 15 minutes, 36 cycles of 94°C for 40 seconds, 66°C for 80 seconds, 72°C for 80 seconds, and 1 cycle of 72°C for 8 min, with the product remaining at 4°C ad infinitum. The amplified 397 bp fragment was analyzed by 2% agarose gel electrophoresis (Invitrogen) and ethidium bromide staining (Invitrogen). The PCR product (7.0 μ L) was incubated at 65°C with 0.7 μ L of *Taq I* Fast Digest (1 U, Fermentas) and 1.34 μ L of enzyme buffer (10x, Fermentas) for 20 minutes. After an electrophoresis run of 30 minutes at 100 volts in 2% agarose gel (Invitrogen), the fragments were viewed using ethidium bromide staining. The PCR product corresponding to the *ADA*1* allele (G22) was then cleaved into two fragments: a 245 bp fragment and a 152 bp fragment. The *ADA*2* allele (22A) was identified by the absence of the *Taq I* restriction site.

Statistical analysis

The GraphPad InStat computer program version 3.06 was used for all statistical calculations. Fisher's exact test was used to detect differences in the distribution of *ADA* genotypes and alleles with respect to RSA; a 5% alpha error was considered acceptable. The differences between mean values for categorical data were calculated by the unpaired t test. Odds ratios and 95% confidence intervals were also calculated. The chi-square test was applied to compare the overall frequencies of *ADA* genotypes and verify whether the distribution of the *ADA* genotypes was in Hardy-Weinberg equilibrium using the Online Encyclopedia for Genetic Epidemiology studies (OEGE) (<http://www.oege.org/software/hwe-mr-calc.shtml>).

RESULTS

This study evaluated women with (G1) and without (G2) a history of RSA. There were statistically significant differences with respect to the mean age (G1: 31.9 ± 5.7 vs. G2: 29.2 ± 5.8 ; $p=0.0001$) and average number of pregnancies (G1: 4.6 ± 1.5 vs. G2: 3.5 ± 0.9 ; $p=0.0001$). The mean number of spontaneous abortions in G1 was 2.7 (± 0.8), ranging from 2 to 6, and the average numbers of live births in G1 and G2 were 0.5 (± 0.3) and 2.6 (± 1.0), respectively ($p=0.0001$).

The identification of *ADA* genotypes was accomplished based on the electrophoretic profile of the 397 bp fragment of exon 1 of the *ADA* gene after digestion with the *Taq I* enzyme (Figure 1). The *ADA*1/*1* and *ADA*1/*2* genotypes were found in G1 and G2, but just one patient with the *ADA*2/*2* genotype was observed in G2 (Table 1). The distributions of the *ADA* genotypes were found to be in Hardy-Weinberg equilibrium in G1 ($\chi^2=0.26$, DF 1) and G2 ($\chi^2=0.57$, DF 1). The frequencies of the *ADA*1/*1*, *ADA*1/*2* and *ADA*2/*2* genotypes were similar and showed no statistically significant differences ($p=0.6771$, $\chi^2=0.78$; DF 2). The differences remained insignificant even when the

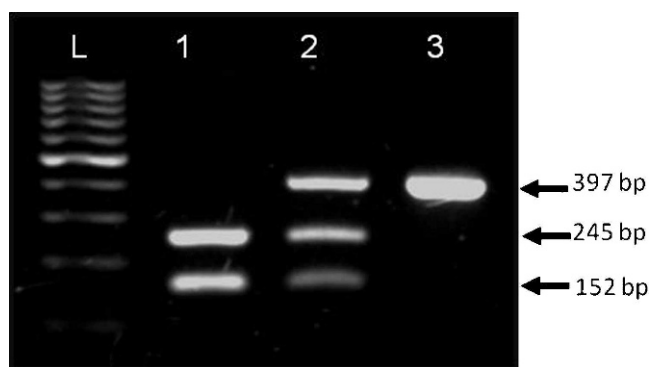


Figure 1 - The electrophoretic profile of the fragment of 397 base pairs (bp) of exon 1 of the *ADA* gene after digestion with the *Taq* I enzyme showing the *ADA**1/*1 (1), *ADA**1/*2 (2) and *ADA**2/*2 (3) genotypes and the marker (L) of 100 base pairs.

comparison between G1 and G2 was restricted to the *ADA**1/*2 and *ADA**2/*2 genotypes ($p=0.8342$, OR=1.177, 95% CI=0.536-2.586). Although the frequencies of the *ADA**1 and *ADA**2 alleles were different in G1 and G2, the difference was not statistically significant ($p=0.7050$, OR: 1.237, 95% CI: 0.578-2.646).

A comparative analysis of the frequencies of the *ADA**1 and *ADA**2 alleles when controlling for age revealed statistically significant differences between the G1 and G2 groups (Table 2).

The data for G1, grouped according to the *ADA* genotypes (*ADA**1/*1 and *ADA**1/*2), are shown in Table 3. There were no statistically significant differences in the mean age, average number of pregnancies, number of live births or comorbidities between women with the *ADA**1/*1 genotype and those with the *ADA**1/*2 genotype. Controlling for comorbidities, these analyses were not statistically significant (diabetes: OR: 0.385, CI 95%: 0.045-3.297, $p=0.3601$; hypertension: OR: 1.817, CI 95%: 0.235-14.017, $p=1.000$; polycystic ovarian syndrome: OR: 4.120, CI 95%: 0.243-69.710, $p=0.3984$; uterine malformation: OR: 1.411, CI 95%: 0.080-24.737, $p=1.000$; antiphospholipid antibodies: OR: 1.046, CI 95%: 0.132-8.241, $p=1.000$; endometriosis: OR: 0.569, CI 95%: 0.159-2.031, $p=0.4198$).

DISCUSSION

The aim of this study was to investigate the association between the G22A polymorphism of the *ADA* gene in Brazilian women with a history of RSA. Because much of the Brazilian population has a clear European ancestry,¹² we

postulated that the *ADA* G22A genetic polymorphism might be associated with RSA in Brazilian women. The series of this study is representative of the population of the northwestern region of São Paulo State, which is chiefly composed of Italian, Spanish, Portuguese, and African descendants.¹⁴ To avoid possible bias, a control group was formed of women who had at least two successful pregnancies and no history of miscarriages.

The women who had experienced RSA who were enrolled in this study (G1) had a higher mean age compared to those who had not (G2). This difference may result from the fact that women without risk factors for spontaneous abortions achieve reproductive success and their desired number of children at an earlier age, whereas women with reproductive problems are encouraged to persist longer in their attempts to conceive and reproduce.¹⁵ Moreover, older age is a risk factor for RSA.^{1,3} This observation is supported by data from a recent demographic survey carried out in Brazil, which showed that maternal age of higher than 35 years contributes to an increased prevalence of RSA.¹⁶

In the present study, women who had experienced RSA became pregnant a significantly higher number of times than those who had not. Spontaneous abortions were confirmed based on clinically recognized pregnancy losses before 20 weeks of gestation; this condition has great predictive value for establishing the occurrence of unsuccessful pregnancies.^{1,3} Women who experience RSA persist in attempts to become pregnant even if the pregnancy does not often reach full term. Moreover, there is evidence that some early fetal losses are not clinically recognized in women with a predisposition to RSA because these pregnancies do not last long enough to be characterized as such. These fetal losses are not included in the statistics, and the rate of spontaneous abortions is therefore underestimated.¹⁷

Several women in G1 gave birth to live babies, but the average number of births was lower than those in G2. This finding is consistent with the finding that women at low risk for RSA are more likely to produce children. Moreover, in women at risk for RSA, the chance of spontaneous abortions increases with each fetal loss.^{18,19} It seems that reproductive success is achieved at a younger age, especially when mothers have no risk factors for RSA, contributing to a greater number of live births in this group.^{16,19}

The frequencies of *ADA* genotypes and the *ADA**1 and *ADA**2 alleles were similar between G1 and G2 and were not associated with the occurrence of RSA. Hence, from a preliminary overview, the results presented in this study are different from those observed for Italian women in whom

Table 1 - The frequencies of *ADA* genotypes and alleles in women with (G1) and without (G2) a history of recurrent spontaneous abortions.

| ADA genotypes | G1 (N = 129) | | G2 (N = 182) | | OR* | CI 95% | p-value** |
|------------------|--------------|------|--------------|------|-------|----------------|-----------|
| | N | % | N | % | | | |
| <i>ADA</i> *1/*1 | 118 | 91.5 | 164 | 90.2 | 1.177 | 0.536 – 2.586 | 0.8435 |
| <i>ADA</i> *1/*2 | 11 | 8.5 | 17 | 9.3 | 0.904 | 0.408 – 2.003 | 0.8436 |
| <i>ADA</i> *2/*2 | 0 | 0.0 | 1 | 0.5 | 0.426 | 0.017 – 10.552 | 1.0000 |
| Alleles | | | | | | | |
| <i>ADA</i> *1 | 247 | 95.7 | 345 | 94.8 | 1.237 | 0.578 – 2.646 | 0.7050 |
| <i>ADA</i> *2 | 11 | 4.3 | 19 | 5.2 | | | |

*Unadjusted odds ratio.

**Calculated by Fisher's exact test.

Table 2 - A comparison of the frequencies of *ADA* alleles and genotypes in women with (G1) and without (G2) RSA aged 35 years or younger and those older than 35 years of age.

| Genotypes | G1 (N = 129) | | G2 (N = 182) | | OR* | CI 95% | p-value** |
|-----------------|--------------|------|--------------|------|--------|----------------|-----------|
| 35 or younger | N = 85 | % | N = 155 | % | | | |
| <i>ADA*1/*1</i> | 76 | 89.4 | 146 | 94.2 | 0.5205 | 0.198 – 1.366 | 0.2042 |
| <i>ADA*1/*2</i> | 9 | 10.6 | 9 | 5.8 | | | |
| Alleles | | | | | | | |
| <i>ADA*1</i> | 161 | 94.7 | 301 | 97.1 | 0.5349 | 0.208 – 1.374 | 0.2126 |
| <i>ADA*2</i> | 9 | 5.3 | 9 | 2.9 | | | |
| Older than 35 | (N = 44) | % | (N = 27) | | | | |
| <i>ADA*1/*1</i> | 42 | 95.5 | 18 | 66.7 | 10.500 | 2.059 – 53.539 | 0.0019 |
| <i>ADA*1/*2</i> | 2 | 4.5 | 8 | 29.6 | 0.1131 | 0.021 – 0.584 | 0.0050 |
| <i>ADA*2/*2</i> | 0 | 0.0 | 1 | 3.7 | 0.1985 | 0.007 – 5.055 | 0.3803 |
| Alleles | | | | | | | |
| <i>ADA*1</i> | 86 | 97.7 | 44 | 81.5 | 9.773 | 2.051 – 46.573 | 0.0012 |
| <i>ADA*2</i> | 2 | 2.3 | 10 | 18.5 | | | |

* Adjusted odds ratio.

** Calculated by Fisher's exact test.

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the *ADA*2* allele was associated with a lower risk of RSA.^{10,11}

Different factors may contribute to the differences observed. It is possible that the *ADA*2* allele has a low frequency in the Brazilian female population as a result of ethnic mixing and that its protective effect is overshadowed by other determinants of RSA. In fact, population analysis revealed that the frequency of the *ADA*2* allele is 0.12 in Caucasians and varies between 0.03 and 0.04 in African descendants.^{7,20} The frequencies of the *ADA*2* allele found in both groups of this study were lower than those reported for Caucasians but very close to those observed in African descendants. Because much of the population of the northwestern region of São Paulo State has African ancestry,¹⁴ this characteristic could contribute to the reduced frequency of the *ADA*2* allele in the casuistic analyzed in this study.

The frequency of the *ADA G22A* genetic polymorphism in different Brazilian regions has been little explored. A recent study found that the frequency of the *ADA*2* allele in healthy men and women in the State of Rio Grande do Sul is 11.7%.²¹ This percentage is approximately twice that found in the present study, but the ethnic background of the population of southern Brazil is different from that of the northwestern region of São Paulo State.^{14,22,23} It is possible that the frequency of the *ADA*2* allele among Brazilians is influenced by ethnic and racial admixture. Investigations focusing the *ADA G22A* polymorphism in other regions of Brazil could confirm this proposition.

The *ADA* genotypes and alleles were compared between the G1 and G2 groups when controlling for maternal age because this variable was found to be associated with the risk for RSA. The results did not reveal statistically significant differences in the frequency of the *ADA*2* allele among those aged 35 years or younger, whereas an association between this allele and low risk for RSA was observed for women older than 35 years of age.

The role of the *ADA*2* allele in reducing the risk of RSA is not completely understood. The presence of an *ADA*2* allele reduces *ADA* enzyme expression to between 15 and 20% of that found in the homozygous *ADA*1/*1* genotype, which enables an increase in adenosine levels.⁷ Consequently,

carriers of at least one *ADA*2* allele have higher levels of circulating and intracellular adenosine.²⁴

Because adenosine acts as a hormone that regulates blood flow, neurotransmission and platelet aggregation and is a potent vasodilator,^{25,26} its presence in the uterus and the placenta could contribute to a reduced rate of early loss of zygotes or fetuses, thus protecting *ADA*2*-carrying women against RSA.^{10,11} This protection may be especially relevant for women older than 35 years of age. Additionally, the increased adenosine resulting from the *ADA*2* allele could contribute to vascular integrity, thus increasing uterine and placental blood flow.⁹ Therefore, evaluations of *ADA* levels at different times during the gestational period among women carrying distinct *ADA* genotypes could help to clarify the potential effects of the *ADA*2* allele during successful pregnancies and shed additional light on the biological and clinical impact of this enzyme on RSA and in assisted reproduction.

Different studies have observed low frequencies of the *ADA*2* allele in couples with sterility problems, women suffering from RSA, those with great variability in gestation time and those who have low birth weight newborns.^{10,11} Because this enzyme has an important role in modulating the immune response, a reduction in its expression seems to affect the fertility rate of women older than 35 years of age.

To assess the differential effects of the *ADA*1* and *ADA*2* alleles on miscarriages, the comorbidities of pregnant women in G1 with the *ADA*1/*1* and *ADA*1/*2* genotypes were compared. There were no statistically significant differences in mean age, the average numbers of pregnancies and spontaneous abortions, the average number of live births, diabetes (including pregnancy-related and type II diabetes), hypertension, polycystic ovarian syndrome, uterine malformations, antiphospholipid antibodies or endometriosis. Therefore, the *ADA*1* and *ADA*2* alleles, when controlled for comorbidities, are not associated with the presence or absence of RSA. These comorbidities are associated with miscarriages, but their influence may be independent of the *ADA* alleles and genotypes.

The small number of *ADA*1/*2* women in G1, which reduces the power of the test, does not allow an analysis of whether either allele provides a biological advantage with

Table 3 - The demographic characteristics, reproductive histories and comorbidities of women who had suffered two or more recurrent spontaneous abortions according to the ADA*1/*1 and ADA*1/*2 genotypes (N = 129).

| Characteristics | ADA*1/*1 (N = 118) | | ADA*1/*2 (N = 11) | | p-value* |
|-----------------------------|--------------------|------|-------------------|------|------------|
| Mean age (SD) | 32.1 (± 5.7) | | 30.0 (± 5.1) | | 0.2301 |
| Pregnancies (SD) | 4.7 (± 1.5) | | 3.6 (± 1.1) | | 0.2417 |
| Live births (SD) | 0.7 (± 0.5) | | 0.5 (± 0.3) | | 0.1953 |
| Miscarriages (SD) | 2.7 (± 0.8) | | 2.5 (± 0.6) | | 0.6765 |
| Comorbidities | N | % | N | % | p-value ** |
| Diabetes | 3 | 1.9 | 1 | 9.1 | 0.3030 |
| Hypertension | 16 | 13.3 | 1 | 9.1 | 1.0000 |
| Polycystic ovarian syndrome | 18 | 15.2 | 0 | 0.0 | 0.3607 |
| Uterine malformation | 6 | 2.8 | 0 | 0.0 | 1.0000 |
| Antiphospholipid antibodies | 9 | 8.5 | 1 | 9.1 | 1.0000 |
| Endometriosis | 14 | 9.5 | 3 | 27.3 | 0.1602 |

*Calculated by an unpaired t test.

**Calculated by Fisher's exact test.

respect to early fetal loss as established by other determinants of RSA.

In summary, the data of this study show that the risk for RSA increases with maternal age and that the ADA*2 allele of the ADA gene is associated with low risk of RSA among older women.

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AUTHOR CONTRIBUTIONS

Nunes DPT and Spegiorin LCJF contribute equally like first author. Nunes DPT and Brandão de Mattos CC performed the genomic analysis. Spegiorin LCJF, Oliani AH and Vaz-Oliani DCM are MD and selected the casuistic and provided samples. de Mattos LC conceived the study and prepared the manuscript.

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